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Isolation and Purification of Enterocin E-760 with Broad Antimicrobial Activity against Gram-Positive and Gram-Negative Bacteria▽

J. E. Line,[1]* E. A. Svetoch,[2] B. V. Eruslanov,[2] V. V. Perelygin,[2] E. V. Mitsevich,[2] I. P. Mitsevich,[2] V. P. Levchuk,[2] O. E. Svetoch,[2] B. S. Seal,[1] G. R. Siragusa,[1] and N. J. Stern[1]

USDA-ARS, Russell Research Center, Poultry Microbiological Safety Research Unit, Athens, Georgia,[1] and State Research Center for Applied Microbiology and Biotechnology, Obolensk, Russian Federation[2]

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Strain NRRL B-30745, isolated from chicken ceca and identified as *Enterococcus durans*, *Enterococcus faecium*, or *Enterococcus hirae*, was initially identified as antagonistic to *Campylobacter jejuni*. The isolate produced a 5,362-Da bacteriocin (enterocin) that inhibits the growth of *Salmonella enterica* serovar Enteritidis, *S. enterica* serovar Choleraesuis, *S. enterica* serovar Typhimurium, *S. enterica* serovar Gallinarum, *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Morganella morganii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, *Campylobacter jejuni*, and 20 other *Campylobacter* species isolates. The enterocin, E-760, was isolated and purified by cation-exchange and hydrophobic-interaction chromatographies. The proteinaceous nature of purified enterocin E-760 was demonstrated upon treatment with various proteolytic enzymes. Specifically, the antimicrobial peptide was found to be sensitive to beta-chymotrypsin, proteinase K, and papain, while it was resistant to lysozyme and lipase. The enterocin demonstrated thermostability by retaining activity after 5 min at 100°C and was stable at pH values between 5.0 and 8.7. However, activity was lost below pH 3.0 and above pH 9.5. Administration of enterocin E-760-treated feed significantly ($P < 0.05$) reduced the colonization of young broiler chicks experimentally challenged and colonized with two strains of *C. jejuni* by more than 8 $\log_{10}$ CFU. Enterocin E-760 also significantly ($P < 0.05$) reduced the colonization of naturally acquired *Campylobacter* species in market age broiler chickens when administered in treated feed 4 days prior to analysis.

Microorganisms produce a variety of compounds which demonstrate antibacterial properties. One group of these compounds, the bacteriocins, consists of relatively small bactericidal peptides. The widespread occurrence of bacteriocins in bacterial species isolated from complex microbial communities, such as the intestinal tract, oral surfaces, or other epithelial surfaces, suggests that they may have a regulatory role in terms of population dynamics within bacterial ecosystems. Bacteriocins are defined as compounds produced by bacteria that have a biologically active protein moiety and bactericidal action (51). In recent years, a renewed interest in bacteriocinlike activities has led to the discovery, isolation, and purification of bacteriocins from both gram-negative and gram-positive organisms (28). They are now being considered for a variety of antimicrobial uses in foods and medicine (17, 41).

Lactic acid bacteria (LAB) are among the most well known and investigated producers of microbial antagonists. These include the well-characterized bacteriocins (6, 30, 55), potential bacteriocinlike substances (57), and other antagonists not necessarily related to bacteriocins (24, 36, 44). The LAB are gram-positive, non-spore-forming, catalase-negative organisms devoid of cytochromes. They are anaerobic but are aerotolerant, fastidious, acid tolerant, and strictly fermentative, with lactic acid as the major end product of sugar fermentation. Lactic acid-producing bacteria include *Lactobacillus* species, *Bifidobacterium* species, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactococcus lactis*, *Streptococcus cricetus*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *Sporolactobacillus inulinus*, and *Streptococcus thermophilus*, etc. These species are of particular interest in terms of the widespread occurrence of bacteriocins within the group and are also in wide use throughout the fermented dairy-, food-, and meat-processing industries. Their role in the preservation and flavor characteristics of foods has been well documented (14).

Most of the bacteriocins produced by LAB are active only against LAB and other gram-positive bacteria. A cursory search of the pertinent literature reveals well over a thousand research articles published in the past 40 years describing the isolation of bacteriocins. Of these, only a small percentage display antibacterial activity toward more phylogenetically distant gram-positive bacteria and, under certain conditions, gram-negative bacteria. In general, bacteriocins are thought to exhibit an antagonistic activity against bacteria genetically closely related to the producer strain; however, a few exceptions with broad-spectrum activity have been described. De Kwaadsteniet et al. (18) described the characterization of a 3,944-Da bacteriocin produced by *Enterococcus mundtii* with activity against gram-positive and gram-negative bacteria. These researchers also listed seven other bacteriocins with broader-spectrum activities, including specific bacteriocins produced by *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Streptococcus thermophilus*, *Enterococcus faecalis*, and *Lactococcus lactis* (1, 10, 27, 32, 35, 53, 54).

* Corresponding author. Mailing address: PMSRU, USDA-ARS, Russell Research Center, 950 College Station Rd., Athens, GA 30604. Phone: (706) 546-3522. Fax: (706) 546-3771. E-mail: eric.line@ars.usda.gov.

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Kuwano and others (33) suggest that nisin Z, produced by *Lactococcus lactis*, utilizes two distinct, salt-dependent mechanisms of antibacterial activity against gram-positive and gram-negative bacteria.

Campylobacter jejuni is a gram-negative, food-borne, human pathogen of primary importance (52). Poultry are frequently contaminated with *C. jejuni* during production, with the majority of commercial U.S. flocks being highly positive for the organism by the time the birds reach market age at approximately 6 weeks of age (46). *C. jejuni* is a commensal organism in poultry, and consequently, colonization with this organism causes no apparent health problems for these birds (7). There are currently no applicable, on-farm interventions for reducing the colonization of poultry with *C. jejuni*. There is a need for effective interventions that may be practically applied in the poultry industry to reduce the colonization of poultry with *C. jejuni* and subsequently reduce consumer exposure to this pathogen. In this paper, we (i) report the isolation and purification of a 5,362-Da bacteriocin produced by an *Enterococcus* species isolated from chicken ceca with broad-spectrum activity against both gram-positive and gram-negative bacteria and (ii) demonstrate the ability of the purified enterocin to reduce the colonization of poultry by *Campylobacter* species.

TABLE 1. In vitro efficacy of enterocin E-760 against *Campylobacter* species strains isolated from chickens in the Russian Federation

Campylobacter sp. strain	MIC of enterocin E-760 (μg/ml)
P 1-R	0.2
P-45	0.4
C. lari R	0.2
LB 6-R	0.1
C/D	0.2
F-2	0.1
P-49	0.4
Ch 3-R	1.6
CR	0.1
Ch-5	0.1
P-57	0.1
P-53	0.1
P-49	0.4
P-78	0.05
P-72	0.05
P-80	0.2
P-88	0.05
P-85	0.05
P/6-2	0.1
P-48	0.05

MATERIALS AND METHODS

Bacterial isolation and identification. LAB, including enterococci and streptococci, were obtained from ceca of healthy broiler chickens in the Russian Federation that were sampled as previously described (47). Cecal materials were suspended in sterile saline solution (pH 7.0), and a 10-fold-diluted suspension was surface plated on enterococcus-selective medium (SRCAMB, Russia). The plates were then incubated at 37°C for 16 to 18 h. Ceca from a total of 376 broiler chickens were analyzed.

Screening for bacteriocin production. Isolated *Enterococcus* and *Streptococcus* strains were evaluated for antimicrobial activity against *Campylobacter jejuni* NCTC 11168 by the agar block method (49). Briefly, approximately 10^7 CFU of each isolate was individually suspended in normal saline, plated on the surface of MRS agar, and incubated for 24 h at 37°C. Agar blocks (diameter, 5 mm) containing growth were aseptically excised from the MRS agar and placed upside down on the surfaces of brucella agar plates supplemented with 5% lysed blood and seeded with $\sim 10^7$ cells of *C. jejuni* NCTC 11168. Plates were incubated under a modified atmosphere (5% O_2, 10% CO_2, 85% N_2) for 24 to 48 h at 42°C. Antagonism of the isolates was evaluated by measuring the diameters of the resulting inhibition zones for *Campylobacter jejuni* growth.

Bacteriocin production and purification. The bacterial strain producing the largest zones of inhibition was isolated and evaluated for bacteriocin production by following the general procedures of Muriana and Luchansky (38). A broth culture of the strain was grown in 6.5 liters of brucella broth (Difco, Detroit, MI) at 37°C for 16 h. The bacteriocin was first purified by cation-exchange chromatography. SP Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) was equilibrated with 20 mM sodium phosphate (pH 4.5), and 1.0 ml was added to 500 ml of the fermentation product in a centrifuge bottle. The bottle was gently agitated, held for 1 h at room temperature, and centrifuged at 20°C for 20 min at 12,000 × *g*. The supernatant was discarded, and the bottle containing the pellet and bacteriocin was filled with a washing solution consisting of 20 mM Na_2HPO_4, pH 4.5, and centrifuged at 20°C for 20 min at 12,000 × *g*. This wash step and centrifugation were repeated once. The supernatant was discarded, and the bottle was then filled with 0.9 M NaCl and gently agitated and incubated at room temperature for 1 h. The bacteriocin was now dissolved in the supernatant, which was again centrifuged at 12,000 × *g* for 15 min at 20°C. The supernatant was transferred to a clean container.

The bacteriocin was further purified by hydrophobic-interaction chromatography. Octyl Sepharose (Amersham Biosciences, Uppsala, Sweden) was equilibrated with 40 mM K_2HPO_4, pH 4.5, and added to the bacteriocin-containing solution at a 1:100 (vol/vol) ratio. The bottle holding the bacteriocin solution and octyl Sepharose was mixed vigorously and incubated at room temperature for 1 h. The bottle was centrifuged at 12,000 × *g* for 20 min at 20°C, and the supernatant was discarded. Washing solution consisting of 20 mM K_2HPO_4, pH 5.5, was added, and the pellet was washed twice and centrifuged as before. Following centrifugation, an elution buffer of 25 mM Tris-HCl, 15 mM K_2HPO_4, pH 4.5, was added to the pellet. The contents of the bottle were centrifuged at 12,000 × *g* for 15 min at 20°C, and the supernatant was transferred to sterile containers for storage. The purified active peptide was designated bacteriocin E-760.

Evaluation of in vitro bacteriocin activity. Target bacteria for assessing the antagonistic activity of the bacteriocin included isolates of *Campylobacter* species and other gram-negative and gram-positive organisms, all of which were obtained from the culture collection at the State Research Center for Applied Microbiology and Biotechnology (SRCAMB), Obolensk, Russian Federation (Tables 1 and 2). The antagonistic activity of the bacteriocin was evaluated by a spot test by creating a series of 1:2 dilutions of the bacteriocin solution in sodium phosphate buffer (pH 7.0). Ten-microliter portions of each dilution were spot plated onto agar previously seeded with cells of the target organisms by the methods of Zheng and Slavik (58). Cultures of *Campylobacter* isolates were grown on brucella agar containing 5% lysed blood and incubated at 42°C for 24 to 48 h under microaerobic conditions. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* were cultured aerobically at 28°C on nutrient agar for 24 to 48 h, and the other strains (Table 2) were cultured on nutrient agar at 37°C for 24 h. The activity of the bacteriocin preparation was expressed in arbitrary units per 1 ml of the most dilute preparation at which a visible zone of inhibition of the growth of culture was observed (26).

Characterization of the bacteriocin by electrophoresis. The molecular mass of the bacteriocin peptide was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25) using a method described previously (49). Briefly, a 15% agarose gel (9 by 12 cm) was prepared and placed in Tris-glycine buffer (pH 8.3). Molecular weight markers (low molecular weight range, 6,500 to 97,000; Amersham Pharmacia Biotech, Piscataway, NJ) were loaded in the first lane of the gel. A second molecular weight marker, insulin chain β (Sigma, St. Louis, MO), was loaded in the second lane, and the purified-bacteriocin-containing solution was loaded in the third lane of the gel. After electrophoresis was performed for 4 h at 400 mA, the gel was fixed with a solution containing 15% ethanol and 1% acetic acid. The gel was washed with distilled water for 4 h. To identify peptide fractions, the gel was stained with a solution containing 0.15% Coomassie brilliant blue R-250 (Sigma, St. Louis, MO) in 40% ethanol and 7% acetic acid. The gel was then sequentially washed with phosphate-buffered saline (pH 7.2) for 1.5 h and deionized water for 3 h. To determine the peptide bands responsible for anti-*Campylobacter* activity, the renatured, stained gel containing the separated peptides was placed in a petri dish and overlaid with semisolid brucella agar (0.75% agar) seeded with cells of *C. jejuni* NCTC 11168. The plate was incubated under microaerobic conditions for 48 h at 42°C. An assessment of peptide bands responsible for activity was based on zones of inhibition of *Campylobacter* growth surrounding the bands.

TABLE 2. Antibacterial activity of enterocin E-760 against various bacteria as determined by a spot test

Gram stain reaction	Bacterial test strain	MIC of enterocin E-760 (μg/ml)
Negative	*S. enterica* serovar Enteritidis 1	0.2
	S. enterica serovar Enteritidis 4	0.4
	S. enterica serovar Enteritidis 204	0.2
	S. enterica serovar Enteritidis 237	0.2
	S. enterica serovar Choleraesuis 434/4	0.4
	S. enterica serovar Choleraesuis 370	0.4
	S. enterica serovar Typhimurium 383/60	0.4
	S. enterica serovar Typhimurium 320	0.2
	S. enterica serovar Gallinarum biovar Pullorum	0.4
	Escherichia coli HB101	0.1
	E. coli C600	0.1
	E. coli O157:H7 Y-63	1.6
	E. coli O157:H7 G-3	1.6
	E. coli O157:H7 OD-3	1.6
	E. coli O157:H7 T-39	1.6
	Yersinia enterocolitica 03	0.1
	Y. enterocolitica 09	0.1
	Y. enterocolitica 04	0.1
	Y. pseudotuberculosis ser 4	3.2
	Y. pseudotuberculosis 14	3.2
	Citrobacter freundii	1.6
	Klebsiella pneumoniae	3.2
	Shigella dysenteriae	0.1
	C. jejuni L4	0.1
Positive	*Staphylococcus aureus*	1.6
	Staphylococcus epidermidis	1.6
	Listeria monocytogenes 9-72	0.1

Characterization of the bacteriocin by isoelectric focusing. The isoelectric point (pI) of E-760 was determined (43). The purified bacteriocin was placed on an isoelectric focusing gel (pH 4.4 to 10.0; Novex, San Diego, CA) and exposed to isoelectric focusing (100 V for 1 h, 200 V for 2 h, and 500 V for 30 min) in an Xcell II mini-cell (Novex). The gel was washed with distilled water for 30 s without fixation and then stained with Coomassie brilliant blue R-250 to determine the pI of the bacteriocin. The peptide fraction responsible for activity was determined by the agar overlay method described above.

Amino acid sequencing. The amino acid sequence of the purified bacteriocin was determined by Edman degradation (39) using a model 491 cLC automatic sequencer (Applied Biosystems, La Jolla, CA) per the manufacturer's instructions. Following the biochemical determination of the primary amino acid sequence, the predicted physical characteristics were analyzed (40) by utilizing Protean software (DNAStar, Madison, WI). The primary amino acid sequence was entered into BLAST (3) to search for peptides with similar sequences.

Mass spectrometry. The molecular mass of the purified bacteriocin was determined by matrix-assisted laser desorption and ionization–time of flight mass spectrometry by using a Voyager-DERP mass spectrometer (PerkinElmer, Wellesley, MA).

Effects of enzymes, temperature, and pH on the antimicrobial activity of the bacteriocin. The influence of enzymes on bacteriocin activity was determined by transferring 10-μl portions of each of the following enzymes (Sigma, St. Louis, MO) to tubes containing 2.0 mg/ml of the bacteriocin: beta-chymotrypsin (100 mg/ml), proteinase K (200 mg/ml), papain (60 mg/ml), lysozyme (75 mg/ml), and lipase (100 mg/ml). After 3 h of incubation at 37°C, the mixture of bacteriocin and enzymes was analyzed for antimicrobial activity by using the spot test described above. Untreated bacteriocin served as the control.

To evaluate thermal stability, a 2-mg/ml bacteriocin solution was boiled in a water bath for 5 min, cooled in an ice bath, and assessed for antimicrobial properties using the spot test described above. To evaluate the effect of pH on the bacteriocin, 2-mg/ml solutions were treated dropwise with 10 mM NaOH or 10 mM HCl to achieve pH values between 3.0 and 10.0. Samples were incubated for 2 h and 24 h at 37°C and for 20 min at 90°C. Following incubation, the pH was adjusted to 7.2 by the addition of 4 mM sterile phosphate buffer, and the antimicrobial activity was determined by the spot test described above.

Dispersion of the bacteriocin into feed. The bacteriocin was dispersed in chicken feed by methods described previously by Stern et al. (48). Briefly, purified bacteriocin (500 ml) was mixed into a 25-ml solution (0.8 mol K_2HPO_4 per liter). This solution was thoroughly mixed with 100 g of ground maize to produce a high-concentration, bacteriocin-treated feed. This feed was mixed with commercial feed to produce bacteriocin-treated feeds having final concentrations of 125 mg, 62.5 mg, and 31.2 mg bacteriocin kg^{-1} feed.

Chicken challenge with *C. jejuni*, bacteriocin treatments, and sampling. An experiment was conducted to demonstrate the in vivo efficacy of bacteriocin-emended feed in reducing *Campylobacter* colonization of young chickens. Day-of-hatch chicks (40) were obtained from a commercial poultry producer and challenged with two strains of *C. jejuni* (B1 and L4) by oral gavage of about 10^6 cells within 24 h of hatching. The chicks were randomly divided among four pens and given free access to feed and water. Beginning on the fourth day after hatching, feed emended with either 125 mg, 62.5 mg, or 31.2 mg bacteriocin kg^{-1} feed was provided to three of the groups. The fourth pen served as the positive control and received bacteriocin-free feed. The chicks were euthanized on day 7 posthatching, and ceca were aseptically removed for *Campylobacter* content analysis by plating of samples on selective agar using methods described previously (49).

A second experiment was conducted to investigate the in vivo efficacy of bacteriocin-treated feed in reducing natural *Campylobacter* colonization in market age broiler chickens. Commercial broiler chickens (39 days old) were obtained from a commercial producer, and fecal samples were analyzed for *Campylobacter* species prior to the placement of 15 birds in individual cages. Control chickens (5) received conventional feed with no added bacteriocin. The treated birds (11) received 125 mg bacteriocin kg^{-1} feed and were given free access to feed and water. Feed consumption was monitored. After 4 days, the birds were euthanized and the ceca were aseptically removed for *Campylobacter* species content analysis by plating of samples on selective agar using methods described previously (49).

Peptide sequence accession number. The sequence data for the peptide of bacteriocin E-760 has been submitted to the UniProt Knowledgebase (Cambridge, United Kingdom) and may be found under accession number P85147.

RESULTS

Bacterial isolation and identification. A total of 376 broiler ceca were analyzed, and 226 bacterial isolates were selected for further analysis of antimicrobial activity. A total of 47 of the 226 isolates evaluated were selected as potential antagonists to *C. jejuni*, and 1 isolate produced the largest zone of *C. jejuni* inhibition surrounding the agar plug. This isolate, initially identified as *Streptococcus criceti* NRRL B-30745, was further characterized by 16S rRNA sequencing (Accugenix, Newark, DE) and identified as an *Enterococcus* species which matched the *E. durans-E. faecium-E. hirae* group of species in the library, normally considered to be a group or complex of very closely related species. Current taxonomy did not allow a single name to be assigned to the organism. The strain is a gram-positive coccus capable of growth between 37 and 45°C. The bacterium is a facultative aerobe and produces circular, regularly shaped, low, convex, grayish colonies with wavy margins about 2 mm in diameter after aerobic incubation at 37°C for 24 h on nutrient agar. The strain degrades lactose, mannitol, ribose, salicin, sorbitol, trehalose, arabinose, and melibiose, and it slightly hydrolyzes raffinose and inulin but does not hydrolyze arginine or esculine. It is not capable of hemolysis, nor does it grow in the presence of 6.5% NaCl, and it is catalase negative. The isolate was deposited under the provisions of the Budapest Treaty with the USDA Agricultural Research Service Patent Culture Collection (National Center for Agricultural Utilization Research, Peoria, IL).

In vitro antimicrobial activity and biochemical characterization of bacteriocin E-760. The in vitro inhibitory activity of bacteriocin E-760 against 20 different *Campylobacter* species

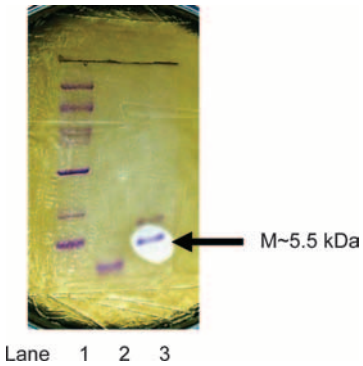

FIG. 1. Direct detection of enterocin E-760 after SDS-PAGE. Following electrophoresis, the gel was overlaid with *C. jejuni* NCTC 11168 in semisolid brucella agar to determine which band(s) corresponds to the antimicrobial activity and molecular weight. Lane 1, molecular mass markers (97, 66, 45, 30, 21.1, 14.3, and 6.5 kDa); lane 2, molecular mass marker for insulin chain β (3.5 kDa). The band in lane 3 (pure bacteriocin E-760), which corresponds to the antimicrobial activity as demonstrated by the zone of growth inhibition (arrow), had a mass (M) of about 5.5 kDa.

isolates selected from chickens in different regions of the Russian Federation is reported in Table 1. The bacteriocin was found to be effective against all 20 strains of *Campylobacter* evaluated by the spot test, with some slight variation in the MICs observed. The lowest MIC measured was 0.05 μg/ml for five of the strains, and the highest MIC was 1.6 μg/ml for a single isolate. The susceptibilities of various other gram-negative and gram-positive bacteria to bacteriocin E-760 were also evaluated by the spot test, and the results are presented in Table 2. Bacteriocin E-760 was found to be inhibitory to a wide spectrum of bacteria and to have a high level of antagonistic activity.

SDS-PAGE, amino acid sequencing, and mass spectroscopy. Purified bacteriocin E-760 was characterized by SDS-PAGE analysis and isoelectric focusing. SDS-PAGE analysis revealed a single peptide fraction which produced a clear zone of inhibition of *C. jejuni* growth, as shown in lane 3 of Fig. 1. Lanes 1 and 2 of the SDS-PAGE gel contained molecular weight markers which demonstrated the active peptide band to have a molecular mass of about 5,500 Da. Isoelectric focusing revealed the active peptide to have a pI of about 9.5.

The purified bacteriocin E-760 was found to contain a peptide comprised of 62 amino acid residues. The result of the amino acid sequencing of the bacteriocin revealed the following peptide: N terminus-NRWYCNSAAGGVGGAAVCGLA GYVGEAKENIAGEVRKGWGMAGGFTHNKACKSFPGS GWASG-C terminus. The peptide was composed of 19.35% charged residues (RKHCE), of which 4.8% were acidic (E) and 11.3% were basic (KR); 46.77% polar residues (NCST YG); and 33.88% hydrophobic residues (AILFWVP), with no aspartate (D) or glutamine (Q). Computer analyses of the E-760 amino acid sequence resulted in a peptide consisting primarily of amphipathic regions at the N-terminal portion and in the interior portion of the peptide with a predicted pI of 8.7. This structure was as predicted for other membrane-disrupting cationic class II antibacterial peptides (22). The molecular mass of the active peptide was confirmed by matrix-assisted laser desorption and ionization–time of flight mass spectrometry and averaged 5,362 Da.

TABLE 3. Effects of enzymes and temperature on the antimicrobial activity of enterocin E-760

Treatment	Activity[a]
Beta-chymotrypsin	−
Proteinase K	−
Papain	−
Lysozyme	+
Lipase	+
100°C, 5 min	+

[a] Activity was determined by a spot test, with *C. jejuni* strain L4 as the indicator strain, after treatment with enzymes or exposure to temperature. +, presence of activity; −, absence of activity.

Effect of enzymes, temperature, and pH on the antimicrobial activity of bacteriocin E-760. The proteinaceous nature of purified bacteriocin E-760 was demonstrated upon treatment with various proteolytic enzymes. The bacteriocin was degraded by beta-chymotrypsin, proteinase K, and papain but was resistant to lysozyme and lipase (Table 3). The bacteriocin demonstrated thermostability by retaining activity after 5 min at 100°C and was stable at pH values between 5.0 and 8.7; however, activity was lost at pH 3.0 and above pH 9.5 (Table 4).

In vivo efficacy of bacteriocin E-760 against *C. jejuni* in week-old broiler chicks. Administration of bacteriocin E-760-treated feed significantly ($P \leq 0.05$) reduced the colonization of young broiler chicks experimentally challenged with two strains of *C. jejuni* (Table 5). *C. jejuni* was not detected in any of the three treated groups of chicks by the methods employed, whereas the untreated control chicks were colonized with about 4×10^8 CFU. This represents a >8-$\log_{10}$ reduction in *Campylobacter* populations in the treated chicks.

In vivo efficacy of bacteriocin E-760 against *Campylobacter* species in market age broiler chickens. Bacteriocin E-760 was also effective at significantly ($P \leq 0.05$) reducing the colonization of naturally acquired *Campylobacter* species in individually housed, market age broiler chickens (Table 6). The untreated control birds were all colonized with an average of 6.17 $\log_{10}$ CFU of *Campylobacter* species g^{-1} cecal contents. *Campylobacter* species were detected in only 1 of the 10 treated birds given free access to feed containing 125 mg bacteriocin kg^{-1} feed for 4 days prior to analysis.

TABLE 4. Effect of pH on the activity of enterocin E-760

pH	Activity determined by a spot test with *C. jejuni* strain L4 after indicated treatment[a]		
	90°C, 20 min	37°C, 2 h	37°C, 24 h
3.0	−	−	−
5.0	+	+	+
6.0	+	+	+
7.0	+	+	+
8.7	+	+	+
9.5	−	−	−
10.0	−	−	−

[a] +, presence of activity; −, absence of activity.

TABLE 5. In vivo efficacy of enterocin E-760-emended feed in reducing the colonization of broiler chicks experimentally challenged with two strains of *C. jejuni*

Treatment group[a]	No. of chicks	Mean chick wt (g)	Mean $\log_{10}$ no. of CFU g^{-1} cecal content	
			C. jejuni[b]	*Lactobacillus* spp.
Control	10	112.5	8.6	>9.0
125 mg E-760 kg^{-1} feed	10	107.2	ND	>9.0
62.5 mg E-760 kg^{-1} feed	10	113.2	ND	>9.0
31.2 mg E-760 kg^{-1} feed	10	112.2	ND	>9.0

[a] All groups were challenged with approximately 6.0 $\log_{10}$ CFU *C. jejuni* strains B1 and L4 by oral gavage on the day of hatching and were given free access to standard broiler feed. The treated groups were given free access to feed emended with bacteriocin E-760 at the specified rates for 3 days beginning the fourth day after hatching. The control group was given standard feed with no enterocin E-760.

[b] ND, no organisms were detected at the limit of 2.0 $\log_{10}$ CFU g^{-1}.

DISCUSSION

Enterococci are widespread in nature and are readily isolated from dairy products (21), vegetables (56), and the gastrointestinal tracts of mammals (34, 45). It is well established that some enterococcal strains produce bacteriocins (enterocins) which have the ability to inhibit the growth of *L. monocytogenes*, *Staphylococcus carnosus*, *Clostridium perfringens*, *Clostridium botulinum*, and *S. aureus* (5, 8, 11). During the past decade, the number of characterized bacteriocins produced by enterococci has increased (23). Analysis and comparison of the amino acid sequences of enterocin E-760 peptides reported herein reveal that from residues 25 through 43 it had a 71% identity with enterocin P. Enterocin P is a pediocinlike bacteriocin isolated from *Enterococcus faecium* that is processed by the general secretory pathway (a *sec*-dependent bacteriocin) and has a broad antimicrobial spectrum against gram-positive pathogenic bacteria (12, 42). The interior portion of the E-760 molecule resembled the enterocin P bacteriocin; however, it lacked the signature YGNGVXC motif in the N-terminal portion of class IIa bacteriocins (17, 20, 41). The extreme N-terminal portion of E-760 more closely resembled lactobin A (16) and amylovorin L (9) isolated from *Lactobacillus amylovorus*. Another similar bacteriocin, gassericin T, which is produced by *Lactobacillus gasseri* (31), contained distinctly identical amino acid sequences in the peptides including the GG VGGA and AVCG motifs. Gassericin T was most similar to a bacteriocin, lactacin F, also produced by various *Lactobacillus* species (2, 37). The locations of the cysteine residues are the interior portions of these molecules, while the E-760 cysteines are more N terminal; in addition, there is one in the C-terminal portion of the sequence. Of interest is the high sequence identity (75%) of residues 31 through 46 of the E-760 molecule with an outer membrane protein of an organism that produces an algicidal peptide (29).

SDS-PAGE cannot accurately estimate the molecular mass of small, hydrophobic bacteriocins like enterocins (5, 13); however, the technique does reveal valuable information regarding the number of peptides responsible for bactericidal activity (23). For this study, a single zone of inhibition was observed for purified enterocin E-760 following SDS-PAGE, indicating the production of a single bacteriocin by the bacterial isolate described herein.

Enterocin E-760 is unique for reported enterocins in its ability to inhibit the growth of a number of campylobacter isolates, as indicated in Table 1. Several other bacteriocins have been demonstrated to inhibit the growth of *Campylobacter* spp. Stern et al. (49) reported a *Lactobacillus salivarius* strain capable of inhibiting a variety of campylobacters, and a *Paenibacillus polymyxa* strain has also been reported to be inhibitory to campylobacters (48, 50). Reuterin, a broad-spectrum antimicrobial compound produced by some strains of

TABLE 6. In vivo efficacy of enterocin E-760-emended feed in reducing the colonization of market age broiler chickens naturally contaminated with *Campylobacter* species

Treatment group[a]	Therapeutic dose of enterocin E-760 (mg $bird^{-1}$)	Live wt of broiler chickens before slaughter (g)	Log_{10} no. of CFU *Campylobacter* spp. g^{-1} fecal sample before treatment	Log_{10} no. of CFU *Campylobacter* spp. g^{-1} cecal content after treatment[b]
Control	0	2,035	6.93	5.15
	0	2,185	6.26	5.81
	0	2,355	4.43	5.51
	0	1,750	4.48	6.04
	0	2,275	5.00	8.36
Mean	0	2,120	5.43	6.17
125 mg E-760 kg^{-1} feed	79.0	2,060	6.92	ND
	58.4	2,065	5.34	ND
	89.0	2,015	4.83	ND
	89.0	1,975	6.98	ND
	80.9	1,755	5.26	ND
	62.8	1,875	5.78	ND
	88.8	2,265	6.67	ND
	88.4	1,985	4.15	ND
	82.8	2,165	5.48	ND
Mean	79.9	2,018	5.71	ND

[a] Broiler chickens (39 days old) naturally colonized with *Campylobacter* species were housed individually. Fecal samples were collected and analyzed for *Campylobacter* species content. Control birds were given free access to standard broiler feed. Treated birds were given free access to feed supplemented with 125 mg bacteriocin E-760 kg^{-1} for 4 days prior to the analysis of *Campylobacter* species in ceca.

[b] ND, no organisms were detected at the limit of 2.0 $\log_{10}$ CFU g^{-1}.

Lactobacillus reuteri, inhibits *C. jejuni* growth (4); however, no other enterococci have previously been reported to produce enterocins inhibitory to the growth of campylobacters or closely related gram-negative microorganisms.

The broad-spectrum antimicrobial activity of E-760 is also unusual among the reported enterocins. The activity observed against gram-negative organisms is particularly unusual and has thus far been reported for only a few bacteriocins produced by LAB (18, 19). The ability of E-760 to inhibit the growth of a number of food-borne pathogens, including salmonellae and campylobacters (Table 2), suggests that this enterocin may be a useful tool in food production and led us to investigate the ability of the enterocin to reduce pathogen populations in vivo. This was demonstrated by the delivery of E-760 in feed, which was efficacious in reducing the colonization of *Campylobacter* spp. in young chicks as well as in market age broiler chickens. While populations of the pathogenic campylobacter were greatly reduced, it was interesting to observe that populations of nonpathogenic LAB in the ceca were unaffected by treatment (Table 5). This is fortunate, as the LAB are thought to be important in maintaining a healthy ecological balance of microorganisms in the gut.

Only two other bacteriocins have been demonstrated to reduce campylobacter colonization in poultry. Cole et al. (15) demonstrated that bacteriocins produced by *Bacillus circulans* and *Paenibacillus polymyxa* reduce cecal campylobacter colonization in turkey poults infected with *C. jejuni*. The bacteriocin purified from *Paenibacillus polymyxa* was also highly effective in reducing the cecal campylobacter colonization of 10-day-old broiler chickens (48). The application of enterocin E-760 to the feed has the potential to be developed as a practical intervention technique for controlling campylobacter colonization in poultry. Economical mass production of the enterocin would be necessary for such an endeavor to be feasible, however. Studies investigating the application of enterocin E-760 in the chickens' water supply are ongoing and will determine if this would be a suitable route of administration. Experiments to optimize the time and duration of enterocin treatment are also being conducted, and the effect on other poultry-associated pathogens, including salmonellae, is being investigated. Large-scale field trials are needed to determine the practical effect of E-760 treatment in a commercial poultry production environment.

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